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Nanoencapsulated Drug-Carrying System for Photodynamic Antimicrobial Chemotherapy (PACT)

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By

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Abstract

The purpose of this study is to develop nanoparticulate drug-carrying systems that are capable of directly delivering photodynamic antimicrobial agents to treat patients with chronic wounds. Chronic wounds are considered as a pandemic health problem. Approximately 1% to 2% of the population in developing countries has experienced a chronic wound during their lifetime. In the United State States, according to the American Academy of Dermatology, the expense of treating chronic wounds constitutes over half of the total cost for all skin diseases, which exceeds $10 billion annually. For the last decade extensive basic science and clinical research in chronic wounds have suggested that pathogenic biofilm is the primary hindrance to the wound healing. Biofilms are highly organized, poly-species bacterial communities living within a protective extracellular matrix that they produce. They are difficult to detect and highly resistant to the host immune system or to antimicrobial elimination. Studies have shown that they can be up to 500 times more resistant to antibiotics than planktonic (unattached, freely living) cells.

Owing to bacterial species within biofilms being exceptionally resistant to many traditional therapies, Photodynamic Antimicrobial Chemotherapy (PACT) can provide an effective alternative for chronic wound treatment. The principle of PACT is derived from traditional photodynamic therapy, which is a technique that uses the combination of light and nontoxic drugs (photosensitizers) to destroy specific targeted cells. After the inactive, nontoxic drug is applied topically or injected, it can only be activated by irradiation with a certain wavelength of light. The light switches on the drug; once the drugs are activated they can produce highly reactive intermediates to destroy the targeted cells without damaging the surrounding healthy tissues. Once the irradiation is removed, the photosensitive drug will return to its stable, non-harmful state. The main advantage of PACT would be that it is very unlikely for bacteria to develop resistance to reactive oxygen species However, the main limitation of this technique would be the uptake kinetics of the photosensitizers in microorganisms. Therefore, the main objective of this study is to find a nanoencapsulated drug-carrying system that can easily penetrate into the polymicrobial species habitat within biofilms.

INTRODUCTION

In 2010, more than 7 million people suffered from chronic wounds and increases by 10% annually.[1] Approximately 80,000 people undergo amputation each year due to wounds that do not heal.[2] Chronic wounds are a pandemic problem that can greatly affect a person's quality of life. The management and treatment cost billions of dollars a year globally. Most health professionals consider a wound to be chronic when it has not healed within 4 to 6 weeks. Surgical textbooks identify chronic wounds are those that do not heal in 3 months.[3] Regardless the length of the time, wounds that do not heal through an orderly progression, halt in a stage dominated by inflammatory processes are considered chronic wounds.

The most common chronic wounds are venous, diabetic, and pressure ulcers. Venous ulcers usually occur in the legs, accounting for about 70% to 90% of chronic wounds. The elderly are affected the most by these ulcers which penetrate deep into the skin and become infected easily. Occasionally, if a venous ulcer persists for a long time, skin cancer could develop at the edge.[4-6] In the United States, 8.3% of the population is diabetic and each year about 65,700 non-traumatic lower-limb amputations are performed in people with diabetic ulcers. In addition, there are about 2.5 million Americans currently hospitalized who are suffering from pressure ulcers (bed-sores).[6,7] The statistical data shows a great need for the treatment of chronic wounds. There are biological and physiological reasons for wounds that are not healing. A primary barrier to healing is the continuing influx of poly-morphonuclear leukocytes (PMNs, a category of white blood cells) from the host blood circulatory system to the open wounds. Activated PMNs release cytotoxic enzymes and inflammatory mediators that can damage host tissues.[8-10] Owing to this continuous influx of PMNs, the healing and the

destructive processes within the chronic wounds are imbalanced; and the main reason for this distress is the presence of biofilms.

Biofilms are highly structured groups of pathogenic microbes living within a protective extracellular matrix that they produce. The extracellular polymeric substance provides physical protection to the microorganisms from its environment. Biofilms in chronic wounds are difficult to detect and highly resistant to the host immune system and antibiotic elimination. Presently, the most common management of biofilm infections is physical removal of the biofilm called surgical debridement.[11] This procedure involves aggressive removal of massive amount of necrotic tissues. Theoretically it would be the preferred method, however, due to the invasive nature it is not always the best option. The life threatening dangers to the patient are tremendous such as anesthesia risk, bleeding, sepsis, and bacteremia.[11.12]

In order to overcome problems associated with the treatment of chronic wounds, non-invasive Photodynamic Antimicrobial Chemotherapy (PACT) can provide an alternative method to heal chronic wound. The principle of PACT is the same as that of traditional photodynamic therapy. It is a non-intrusive technique that uses a combination of light and nontoxic drugs (photosensitizers) to destroy targeted cells. After the inactive, nontoxic drug is applied topically or injected, it can only be activated by irradiation with a certain wavelength of light. Once the drugs are switched on by light, they can produce highly reactive intermediates to destroy the targeted cells without damaging the surrounding healthy tissues. Once the irradiation is removed, the photosensitive drug will return to its stable, non-harmful state.

The main limitation of this technique would be the uptake kinetics of the photosensitizer in microorganisms. In general, neutral, anionic, and cationic photosensitizers can efficiently eliminate Gram-positive bacteria.[13] only hydrophilic cationic photosensitizers

can kill Gram-negative bacteria. The porous cell wall of Gram-positive bacteria allows most photosensitizers to cross. However, the cell envelope (outer membrane) of Gram-negative bacteria forms an effective permeability barrier between the cells and its environment.[14] This has led to intensive research on particulate delivery systems to overcome this situation. Studies have shown that using a nanoemulsion as a carrier for biomedical applications can improve efficacy in solubilizing, protecting, and targeting drugs for specified delivery.[15-17] Therefore one can anticipate such an approach to greatly advance current chronic wound diagnostics and treatments.

EXPERIMENTAL METHODS

Nanoemulsion

The photosensitizers (Active Pharmaceutical Ingredient) used in the study are mostly hydrophobic and less permeable to cross the cell barrier. To intervene this, a nanoemulsion of drug is being prepared for optimal drug delivery. In general, neutral, anionic, and cationic photosensitizers can efficiently eliminate Gram-positive bacteria. Only hydrophilic cationic photosensitizers can kill Gram-negative bacteria. The porous cell wall of Gram-positive bacteria allows most photosensitizers to cross. However, the cell envelope (outer membrane) of Gram-negative bacteria forms an effective permeability barrier between the cells and its environment. This has led to intensive research on particulate delivery systems to overcome this situation. Many studies have shown that using nanoemulsions or nanoparticles as carriers for biomedical applications can improve efficacy in solubilizing, protecting, and targeting microorganisms for specified delivery. Therefore one can anticipate that our approach can greatly advance current chronic wound treatment.

Formulation PACT-3A: The first generation porphyrins tested for photodynamic therapy were based on chemically modified natural hematoporphyrins. They possessed certain limitations such as weak absorption in the phototherapeutic window as well as poor specificity toward malignant and healthy tissues. The second generation of photosensitizers was mainly based on the engineered, synthetic, and semi synthetic porphyrins with various expanded substituents at the pyrrol rings and the methylene bridges. The optical properties for therapy have improved in the second generation photosensitizers but delivery to the target tissue is still a passive process.

In this study, several approaches have been taken in order to improve the direct targeting and to increase reactive species generation of PACT agents. The photosensitizers together with a direct delivery mechanism are known as the third generation of photosensitizers or smart drugs. Also, owing to most porphyrin and phthalocyanine derivatives not being water soluble, oil-in-water nanoemulsion and nanoparticle formulations have been developed to promote drug delivery.

Formulation PACT-3A-1:

I. Dissolve 30 mg copper Phthalocyanine (CuPc) and 4.0 mL of surfynol- 465 in 10 mL of ethyl acetate (organic phase) over low heat with constant stirring.

II. Dissolve 270 mg high molecular weight PEG (Mr > 2000 g/mol) in 50 mL of water (water phase).

III. Add the organic phase into the water phase while vigorously stirring until all the ethyl acetate has evaporated. Sonicate for 15 minutes.

Formulation PACT-3A-2:

I. Dissolve 6 mg of copper Phthalocyanine (CuPc) and 2.0 mL of surfynol 465 in 10 mL of ethyl acetate (organic phase) over low heat with constant stirring.

II. Dissolve 0.2 g of poloxamer- 407 in 20 mL of water (water phase).

III. Add organic phase into water phase while vigorously stirring over low heat until all the ethyl acetate has evaporated.

IV. The solution is then degassed to remove the foam.

V. Sonicate for 15 minutes.

Formulation PACT-3A-3:

I. Dissolve 6 mg (5.5 μmole) of copper Phthalocyanine (CuPc) and 2.0 mL of surfynol- 465 in 20 mL of ethyl acetate (organic phase) over low heat with constant stirring.

II. Dissolve 11 μmole of o-(octadecylphosphoryl)choline in 20 mL of water (water phase).

III. Add organic phase into water phase while vigorously stirring. Keep adding water while stirring and bring the volume to 100 mL.

IV. Keep stirring overnight until all the ethyl acetate has evaporated and bring the volume down to 20 mL.

IV. The solution is then degassed to remove the foam.

V. Sonicate for 15 minutes.

Formulation PACT-3A-4:

I. Dissolve 3 mg of riboflavin and 0.168 g of PLGA in 10 mL of ethyl acetate (organic phase) under low heat with constant stirring.

II. Add 1 mL of Triton X– 100 (HLB 11.6) t0 10 mL of PBS (water phase).

III. Add organic phase into water phase while vigorously stirring over low heat until all the ethyl acetate has evaporated.

IV. Sonicate for 15 minutes.

Stabilizer and Thickening Agent

PEG and poloxamer are well known stabilizers; therefore, no additional stabilizer is needed in some of our formulations. Carbomer, a synthetic high molecular weight polymer of acrylic acid will be used as an additional stabilizer and also as a thickening agent to increase the viscosity of all the formulations.

Singlet Oxygen Study

Singlet oxygen determination has been carried out by UV-Vis spectroscopy (Ocean Optics) with loggerpro-3.6.0 Vernier software. The 9,10-anthracene dipropionic acid (ADPA) photo-bleaching method is used to confirm singlet oxygen generation. ADPA is easily converted to a photo inactive endoperoxide by singlet oxygen. An ADPA solution in deuterium oxide (D_2O) is used as the singlet oxygen acceptor. Mixed solutions of the photosensitizer and ADPA are irradiated with visible light. The reaction progress is monitored by recording the decrease of the 400 nm absorption peak of ADPA verses irradiation time. The intensity of ADPA absorption decreased as the irradiation time increased, which indicates the generation of singlet oxygen.

DNA Study

Drug formulation at different concentrations, pUC19 Plasmid (120 μM), NaCl (5 mM) are added to transparent eppendorf micro tubes. A uniform volume of 20 μL is maintained in all tubes. The tubes are incubated in the dark for 0.5 h with subsequent irradiation of the light samples under visible light in the photoreactor (2.8×10^{-3} W/cm^2 ; 5.0 J/cm^2) for 0.5 h.The dark and light samples are loaded into the gel with gel loading solution (4 μL) and gel electrophoresis (90 V; 1.5 h) is carried out using agarose (2.0%) stained with ethidium bromide in 1X TAE running buffer. The gel is imaged on Gel Doc 200 transilluminator (Bio-Rad, Hercules, CA) with the supercoiled DNA at position 2 and the nicked DNA at position 1. The degree of photocleavage is estimated by the integration of the image intensity at position 1 and position 2 by Quantity One Analysis system software (Bio-Rad, Hercules,CA).

Cell and Bacteria Study

Human skin fibroblasts (Hs-27) from the American Type Culture Collection, cell line CRL-1634 (Manassas, VA) are used in the cell study. The cells were cultured in Dulbecco's Modified Eagle Media (10% fetal bovine serum, 50 μg/mL gentamicin, 4.5 mg/mL glucose, and 4 *mM* L-glutamine). The cells are cultured in BD-Biocoat (60 mm) culture dishes (at 5.0% CO_2 at 37^{o}C in the incubator). The media is washed away from the culture plates using 1X phosphate buffer for the assesment of cytotoxicity and photocytotoxicity of the nanoparticles. Uniform volume of nanoemulsion (test), 1X PBS (blank), and placebo (control) are added to the culture dishes. The culture dishes are incubated for 1.0 h in the dark and in visible light seperately for photocytotoxicity. The supernant from the culture dishes are decanted and washed with 1X PBS. The cells are lysed using cell lysis solution (20 μM sodium laurylsarcosine solution) for approximately 1.0 h on a shaker. The protein content from the lysed culture dishes are

quantified using BCA Protein Assay (Thermopierce). The culture dishes are incubated for an hour and the absorbance intensity of the purple color complex formed is observed at 570 nm in Biotek ELx800 micro plate reader for the dark and light samples.

E coli (Gram negative) and *S. aureus* (Gram positive) are cultured in petri dishes with nutrient broth culture media. The nanoemulsion is added to the bacterial culture and incubated for an hour. Half of the culture remains in the dark and the other half is irradiated for 0.5 h. The absorbance readings of both the irradiated and dark samples are observed on Biotek Epoche Absorbance Plate reader.

RESULTS

Nanoparticles

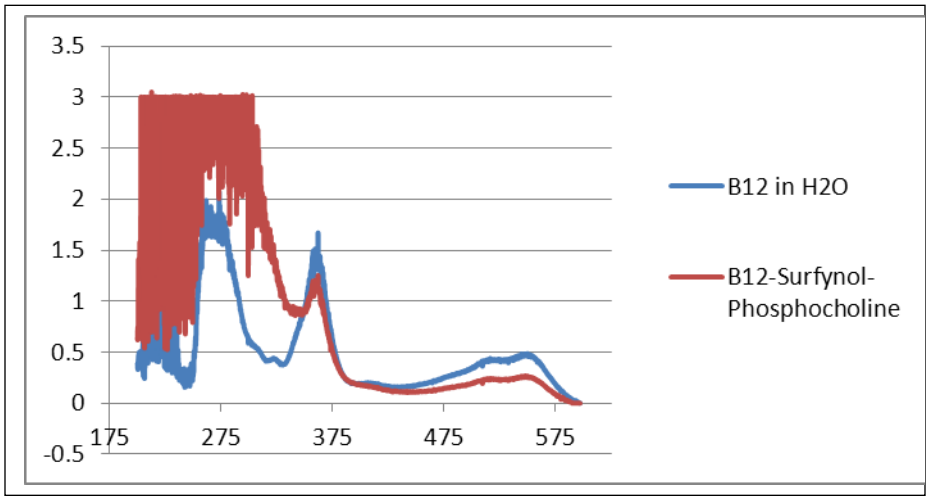

Figure 1

In the above figure, the blue line represents the typical UV-Vis spectrum of aqueous Vitamin B12. it shows a π to π^* transition peak at 272 nm, a *n* to π^* transition peak at 370 nm and a MLCT peak at 570 nm. The red line from the above figure shows decreasing absorption intensity compared to the blue line at the same concentration (50 μM) of Vitamin B12. The graph clearly indicates that vitamin B12 has been entrapped

inside the double coated nanoparticles. The enhancement of UV absorption below 300 nm is due to the absorbtivity of phophocholine which has a λ_{max} of 225 nm.

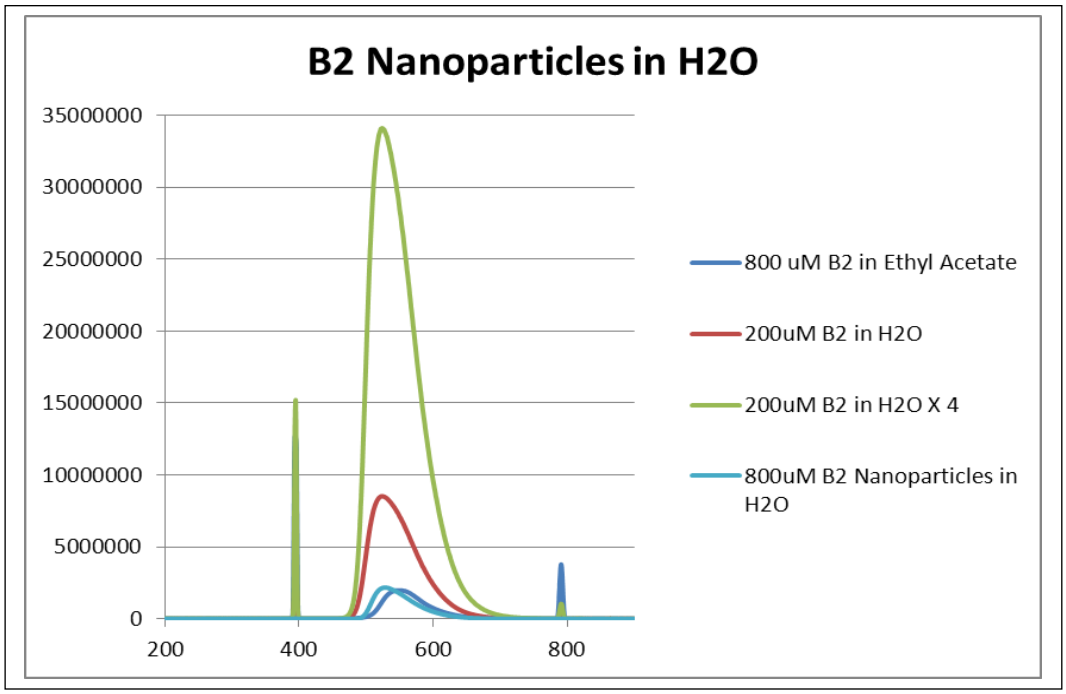

Figure 2

Above figure shows Vitamin B2 is encapsulated inside palmitic acid nanoparticles. It is well known that Vitamne B2 (riboflavin) is not very soluable in water, it reaches the saturation at 200 μM. The red line from above graph shows the fluorescence of 200 μM riboflav in aqueous solution. The green line is arbitrally created to show the intensity of 800 μM riboflavin in water. The dark blue line shows 800 μM riboflavin in ethyl acetate. The light blue line shows 800 μM riboflavin encapsulated inside the nanoparticles then suspended in water. The hypochromic effect of the light blue linecompared to the dark blue line (both 800 μM) indicates the increasing of hydrophilicity. Compare the green

line and the light blue line, it is evident that riboflavin has been encapsulated inside the nanoparticles.

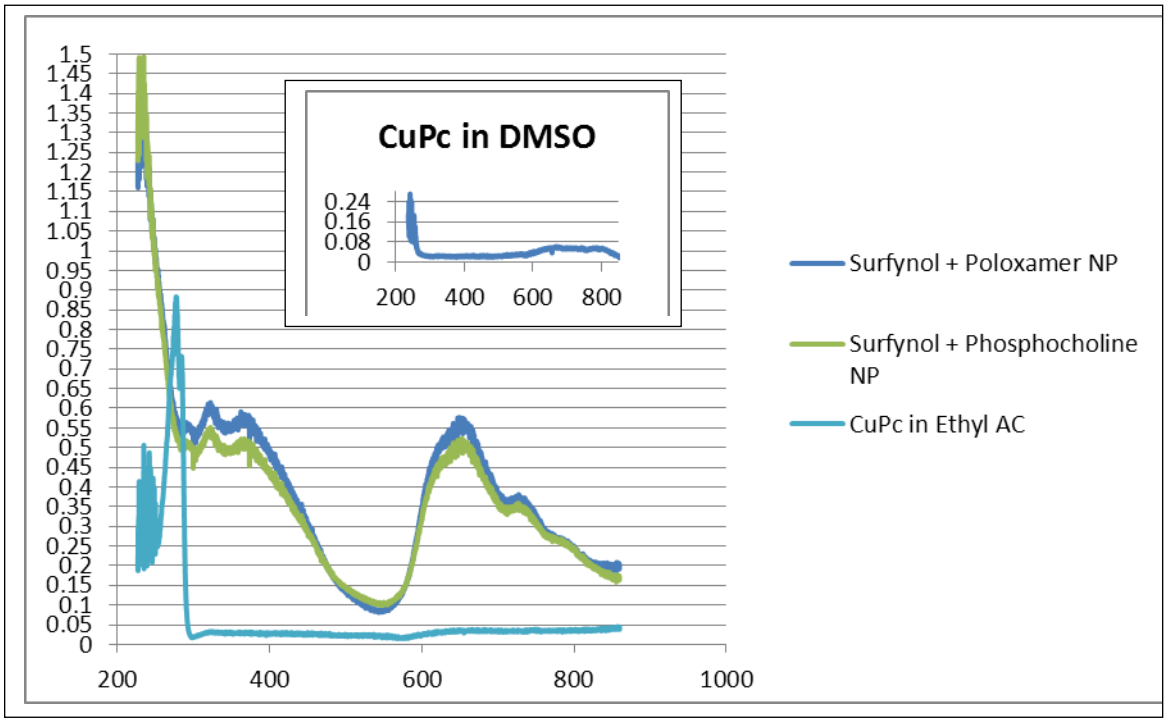

Figure 3

Above figure shows copper phthalocyanine (CuPC) entrapped inside surfynol-poloxamer (dark blue line) and surfynol-phosphocholine (green line) double coated nanoparticles. CuPc is a hydrophobic molecule, when dissolved in a non-polar solvent such as ethyl acetate, the electronic absorption spectra shows only a strong π to π^* peak (the light blue line). Electronic transitions *n* to π^* and MLCT start to take place only when the polarity of the solvent increases. DMSO has a polarity of 7.2, the inset graph shows when CuPc dissolved in DMSO, the absorption spectra starts to show a MLCT band due to the increased excited state dipole moment. The electronic absorption shows very different transitions in the visible region (the dark blue and green lines from the above figure) when CuPc nanoparticles suspended in water (polarity = 10.2) than when it is in the non-polar solvent (the light blue line).

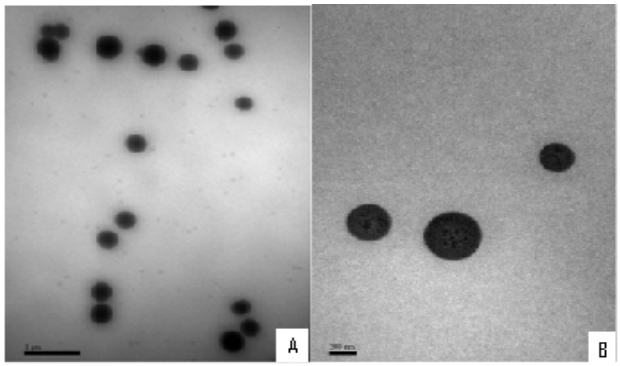

Figure 4

Above figure shows TEM of CuPc encapsulated nanoparticles. (A) Indicated Magnification is X25000 and the Total Magnification is X28800. (B) Indicated Magnification is X60000 and the Total Magnification is X69900.

Singlet Oxygen Study

Singlet oxygen study was also preformed on riboflavin to ascertain its generation over time. Figure 3 shows that after only 0.25 h of irradiation, riboflavin is able to produce a significant amount of singlet oxygen. The sensor used in the singlet oxygen study is 9,10-anthracene dipropionic acid (ADPA). Our result has shown that riboflavin is an excellent source of producing reactive oxygen species when irradiated with low energy visible light.

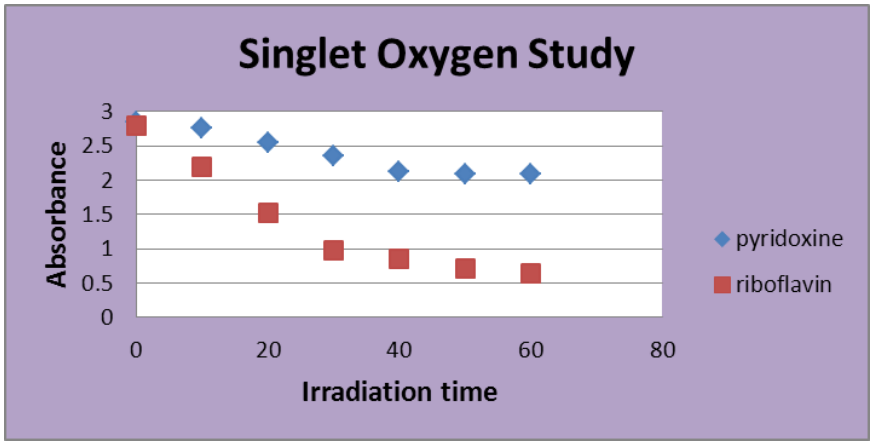

Figure 5

Above figure shows the absorption intensity of ADPA (at 400 nm, in the presence of CuPc) decreased as the irradiation time increased indicates ADPA was converted to an endoperoxide derivative by singlet oxygen.

	Human Skin		E.coli		S. Aureus		Acute Oral
	Dark	Irradiated	Dark	Irradiated	Dark	Irradiated	
Riboflavin	No	2,170 ± 110	197 ±	85 ± 4.20	275 ±	117 ±	>10,000
Phylloquinone	No	18,700 ±	1,100 ±	423 ±	3,270	475 ±	25,000

Table 1. LD50 (lethal dose 50 in mg/Kg) of Riboflavin and Phylloquinone in both dark and irradiated conditions . Acute oral systematic toxicity of both was cited from the official MSDS.

Cytotoxicity and Photocytotoxicity

The cytotoxicity and photocytotoxicity study was executed using human fibroblast skin cells. Table 1 shows that both riboflavin and phylloquinone have no toxic effect on human skin cells in the dark. There is a slight adverse effect when the riboflavin and phylloquinone is irradiated with light, but these numbers are much lower compared to the acute oral toxicity obtained from the MSDS. Furthermore, the reactive oxygen species that are formed when irradiated with visible light have such a short lifetime and can not travel far, so only those cells which are irradiated with light will be effected in the end.

Bacteria Study

The bacterial study of riboflavin and phylloquinone was conducted using *E. coli* and *S. aureus*. A concentration as low as 85 mg/Kg of riboflavin can cause 50% fatality of bacteria after an hour of incubation followed by 0.5 h of irradiation with low intensity light, ~5.0 J/cm^2. These results were compared with the acute oral toxicity of riboflavin and phylloquinone obtained from the official MSDS. Table 1 confirms that at a low

concentration, riboflavin and phylloquinone are able to eradicate bacteria upon irradiation. *S. Aureus* is a gram-positive bacteria that can be easily penetrated by photosensitizers due its porous cell wall. However, *E. Coli* is a gram-negative bacteria which has a outer membrane that is hard to penetrate. Our results indicate our nanoemulsion can easily pass through the bacterial membrane and release the photosensitizers.

DISCUSSION

We have shown that photodynamic antimicrobial chemotherapy (PACT) has the potential to represent an alternative antibacterial, antifungal, and antiviral treatment for drug-resistant organisms. Non-toxic vitamins such as riboflavin and phylloquinone can produce singlet oxygen and free radicals upon irradiation. Our data has shown that riboflavin not only has a great DNA binding constant ($K_b > 10^4\ M^{-1}$), it can also cleave bacterial DNA under the irradiation of the visible light ($\lambda > 395$ nm). Nanoemulsion also has proven to be an effective way for drug delivery. Their photobiological properties and phototoxicity towards prokaryotic cells still needs to be investigated further. In addition, spin-off applications of this work would offer opportunities for other environmental friendly uses such as bioremediating hazardous waste sites, biofiltering industrial water, and forming biobarriers to protect soil and groundwater from contamination.

REFERENCES

[1] A. R. Siddiqui and J. M. Bernstein, Clinics in Dermatology, 28, 519, 2010.

[2] V. Gontcharova, E. Youn, Y. San, R. D. Wolcott and S. E. Dowd, The Open Microbiol. J., 4, 8, 2010.

[3] Bruunicardi F. Chrles Schwartz's principles of surgery 8th ed. New York: McGraw; 2004.

[4] D. T. Ubbink, S. J. Westerbos, D. Evans, L. Land and H. Vermeulen, Cochrane Database Sys Rev., 16, 3, 2008.

[5] E. Ficarelli, G. Bernuzzi, E. Tognetti, O. Bussolati, A. Zucchi, D. Adorni and G. De Panfilis, Dermatol Ther., Jul 21, 13, 2008.

[6] D.M. Smith, D.E. Snow, A. M. Zischkau, J. D. Hanson, R. D. Wolcott, Y. San, J. White, S. Kumar and S. E. Dowd, BMC Med. Geno., 3:41, 2010

[7] B. K. Aronovitch, Nurs. Econ., 17, 263, 1999.

[8] K. Kirketerp-Moller, P. O. Jensen, M. Fazli, K. G. Madsen, J. Pedersen, C. Moser, T. T. Nielsen, M. Givskov and T. Bjarnsholt, 46, 2717, 2008.

[9] V. Falanga, Wound Repair Regen., 8, 347, 2000.

[10] G. S. Schultz, R. G. Sibbald, E. A. Falanga, C. Ayello, K. Dowsett, M. Harding, M. C. Romanelli and W. Vanscheidt, Wound Repair Regen., 11, S1, 2003.

[11] J. Hurlow and P. G. Bowler, Ostomy Wound Manage, 55, 38, 2009.

[12] S. C. Davis, L. Martinez and R. Kirsner, Curr. Diabet. Rep., 6, 439, 2006.

[13] S. Schastak, S. Ziganshyna, B. Gitter, P. Wiedemann and T. Claudepierre, Plos One, 5, 7, 2010.

[14] R. F. Donnelly, P. A. McCarron and M. M. Tunney, Microbiol. Research, 163, 1, 2008.

[15] S. Banfi, E. Caruso, L. Buccafirni, V. Battini, S. Zazzaron, P. Barbieri and V. Orlandi, J. Photochem. Photobiol. B : Biol., 85, 28, 2006.

[16] R. Boyle, Mol. Pharmaceutics, 16 March 2011

[17] C. Duncan, P. I. Hone, R. Evans-Gowing, S. FitzGerald, A. Beeby, I. Chambrier, M. J. Cook, and D. A. Russell, Langmuir, 18, 2985, 2002.